Hox genes specify vertebral types in the presomitic mesoderm

Marta Carapuço,[1] Ana Nóvoa,[1] Nicoletta Bobola,[2] and Moisés Mallo[1,3]

[1]Instituto Gulbenkian de Ciencia, 2780-156 Oeiras, Portugal; [2]Max-Planck Institute of Immunobiology, 79108 Freiburg, Germany

We show here that expression of *Hoxa10* in the presomitic mesoderm is sufficient to confer a *Hox* group 10 patterning program to the somite, producing vertebrae without ribs, an effect not achieved when *Hoxa10* is expressed in the somites. In addition, *Hox* group 11-dependent vertebral sacralization requires *Hoxa11* expression in the presomitic mesoderm, while their caudal differentiation requires that *Hoxa11* is expressed in the somites. Therefore, *Hox* gene patterning activity is different in the somites and presomitic mesoderm, the latter being very prominent for *Hox* gene-mediated patterning of the axial skeleton. This is further supported by our finding that inactivation of *Gbx2*, a homeobox-containing gene expressed in the presomitic mesoderm but not in the somites, produced *Hox*-like phenotypes in the axial skeleton without affecting *Hox* gene expression.

Supplemental material is available at http://www.genesdev.org.

Received January 28, 2005; revised version accepted July 5, 2005.

In vertebrates, the axial skeleton derives from the somites, segmental units organized in pairs on both sides of the developing neural tube (Hirsinger et al. 2000). Somites are formed in a rostro-caudal sequence by the epithelialization of mesenchymal cells at the rostral end of the presomitic mesoderm (Hirsinger et al. 2000). Although somites look morphologically similar, the skeletal elements they form are specific for the axial level at which they are positioned, eventually generating a vertebral formula characteristic for each species. In the mouse, this formula consists of seven cervical, 13 thoracic (which have ribs attached), six lumbar, four sacral, and a variable number of caudal vertebrae (~20) (Burke et al. 1995).

Hox genes are among the major players in the specification of the morphological identity of the vertebrae (Krumlauf 1994). In addition, it has recently been described that some of the *Hox* genes play a global patterning role in vertebral development (Wellik and Capecchi 2003). When and how *Hox* genes determine somitic segmental identity is still an unresolved question. It is generally accepted that a specific combination of *Hox* genes expressed at a particular somitic level determines the axial identity of the resulting structures (Kessel and Gruss 1991; Krumlauf 1994). However, an association between *Hox* somitic expression and mutant phenotypes is not always easy to establish. For instance, axial phenotypes were observed in embryos that recovered appropriate *Hox* expression domains after retarded activation or transient expression in the presomitic mesoderm (Zakany et al. 1997; Kondo and Duboule 1999). Also, for some *Hox* genes, the rostral expression boundaries in the somites seem to lie posterior to their functional domains of activity, an apparent paradox. *Hox* group *10* genes are a good example of such a situation. Recent genetic studies revealed that these genes are functionally relevant up to the thoracic/lumbar transition (Wellik and Capecchi 2003), but published expression patterns for the three *Hox* group *10* genes (*Hoxa10*, *Hoxc10*, and *Hoxd10*) rarely extend to the corresponding somitic level, which in mice corresponds to somite 25 (Burke et al. 1995), and seem to differ according to the embryonic stage analyzed (Kessel and Gruss 1991; Burke et al. 1995; Favier et al. 1996; Zakany et al. 1997). In this study we show that the *Hox10* group expression domain, indeed, corresponds to the genetically defined functional domain but only at the stage at which the somites that correspond to the thoracic/lumbar transition are being formed in the presomitic mesoderm, which suggested that the activity of these genes is functionally relevant at this stage of somite development. We evaluated this hypothesis by comparing the activity of a *Hox10* group and a *Hox11* group gene in the presomitic versus the somitic paraxial mesoderm in transgenic mice. Our results reveal that the relevant function of *Hox* genes is provided in the presomitic mesoderm. We further sustain this conclusion with the finding that *Gbx2*, another homeobox-containing gene expressed in the presomitic and not in the somitic mesoderm, is required for proper patterning of the axial skeleton.

[*Keywords*: Hox genes; axial patterning; Gbx2; presomitic mesoderm; transgenics]
[3]Corresponding author.
E-MAIL mallo@igc.gulbenkian.pt; FAX 351-214407970.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.338705.

Results and Discussion

When we re-evaluated expression of the three *Hox10* group genes at various embryonic stages (Supplemental Material), we found that the anterior expression border of all three *Hox10* group genes is compatible with their reported functional domain (i.e., the thoracic/lumbar transition) only at the stage at which the corresponding somites are being formed. However, as development proceeds, this border recedes to more caudal somites located within the lumbar or sacral areas, or even almost disappears from the somites (Supplemental Material). Considering that all members of the paralog group 10 seem to be functionally equivalent and functionally relevant up to the thoracic/lumbar transition (Wellik and Capecchi 2003), these results could indicate that *Hox10* activity is essential at the stage of somitic formation or shortly thereafter, but it becomes dispensable at later stages of somitic development.

Hoxa10 *activity in the presomitic versus somitic mesoderm*

To test this idea, we used a transgenic approach in which *Hoxa10* was expressed under the control of the *Dll1* pro-

moter (Beckers et al. 2000). This promoter has been shown to be active in the paraxial mesoderm at presomitic stage and in the most recently formed somites, but not at later stages of somite development (Beckers et al. 2000; Cordes et al. 2004). Skeletal analysis of these transgenic embryos revealed striking phenotypes in the axial skeleton (six/six transgenics examined), which varied in intensity with the transgene copy number. In four of these transgenics, a total absence of ribs was evident in the thoracic area (Fig. 1D), consistent with the known activity of *Hox10* group genes in suppressing rib formation (Wellik and Capecchi 2003). The absence of ribs was associated with the absence of sternebrae on the sternum, which appeared as a flat ossified structure (Fig. 1E). In addition, the cervical vertebrae were bigger than those in normal animals, and no cartilaginous fusions between vertebrae at the theoretical sacral level were found (Fig. 1F). In the remaining two transgenic embryos, a fainter effect was found, restricted to the absence of ribs from vertebrae T1, T12, and T13 (Supplemental Material; data not shown). These results indicate that expression of *Hoxa10* under the control of the *Dll1* promoter was able to activate a dominant *Hox10* patterning program in somites at all axial levels. If, as reported, the activity of this promoter is restricted to the presomitic mesoderm and newly formed somites (Beckers et al. 2000; Cordes et al. 2004), these data would imply that *Hox10* activity in the forming somite is sufficient to determine the *Hox10*-dependent morphogenetic program to the somite.

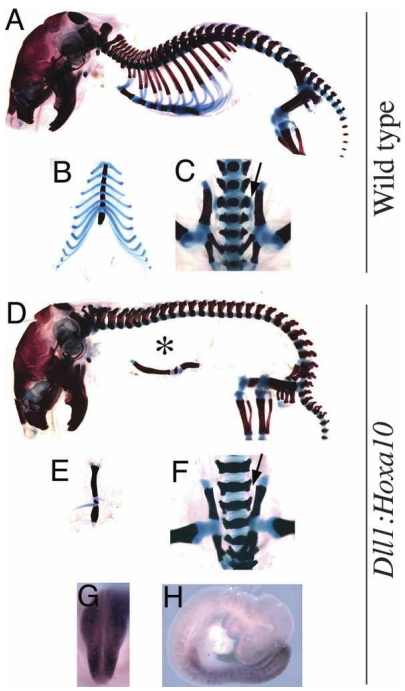

Figure 1. Patterning activity of *Hoxa10* expressed with the *Dll1* promoter. Skeletal staining of wild-type (*A–C*) and *Dll1–Hoxa10* transgenic (*D–F*) newborns. (*A*,*D*) A global view of the animal, after removing the forelimbs for clarity. (*D*) In the transgenic, the ribs are missing from the area labeled with an asterisk. (*B*,*E*) An anterior view of the sternum with the associated cartilaginous part of the ribcage (mostly missing in the transgenic animal). (*C*,*F*) The sacral area. The arrow points to the lateral fusion between sacral vertebrae, missing in the transgenic embryo. (*G*,*H*) An in situ hybridization analysis of *Hoxa10* expression in a *Dll1–Hoxa10* transgenic embryo. (*G*) A dorsal view of the presomitic mesoderm. (*H*) A lateral view of an embryonic day 9.0 (E9.0) embryo.

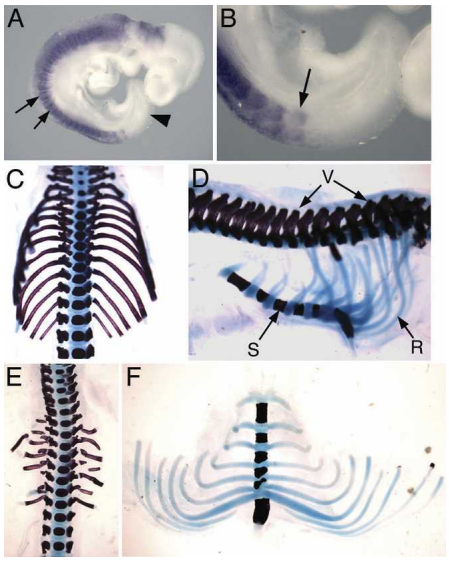

Figure 2. Patterning activity of *Hoxa10* expressed in the somites. (*A*) Analysis of the expression activity of the *sm* promoter. The bacterial *tetR* gene was used as a reporter, and its expression was detected by in situ hybridization. The arrows indicate some somites, and the arrowhead indicates the presomitic mesoderm. (*B*) High-power view of the tailbud region of the embryo shown in *A*. The arrow indicates the last formed somite. (*C*) Ventral view of the thoracic area of a wild-type newborn. The sternum and the cartilaginous area of the ribcage were removed for clarity. (*D*) Lateral view of the thoracic area of an *sm-Hoxa10* transgenic embryo, oriented rostral to the left and ventral at the bottom. The sternum (S), ribs (R), and vertebrae (V) are indicated. (*E*) Ventral view of the thoracic area of an *sm-Hoxa10* transgenic embryo, oriented rostral at the top. The ossified area is strongly malformed. The sternum and the cartilaginous area of the ribcage were removed for clarity and are shown in *F*.

In situ hybridization analysis of *Dll1–Hoxa10* embryos revealed that in some embryos *Hoxa10* expression was not restricted to the presomitic and recently formed somitic paraxial mesoderm, but extended to more rostral somites (Fig. 1G,H), thus opening the possibility that the effect we observed was due to *Hoxa10* expression in the already formed somite. To test this possibility, we generated a new set of transgenic animals (*sm-Hoxa10*) in which *Hoxa10* was expressed under the control of a promoter that is not active in the presomitic mesoderm and becomes activated in the somites after they bud off the presomitic mesoderm (Fig. 2A,B; Supplemental Material). All *sm-Hoxa10* transgenic animals ($n = 8$) had ribs, although their morphology was somewhat abnormal in four of these transgenics (Fig. 2E,F). Normally, the ribs of newborn mice have a dorsal ossified area (about two-thirds of the rib length) articulating with the vertebrae, and a ventral cartilaginous part that attaches to the sternum in the first seven and has a free end in the remaining six (Figs. 1B, 2C). In all four affected *sm-Hoxa10* transgenics, the ossified area was absent or reduced and disorganized (varying from subtle differences to strong malformations as in the embryo shown in Fig. 2). The cartilaginous area was always present and correctly patterned (the first seven were attached to the sternum), but it was consistently much longer than in wild-type littermates, extending further dorsally to attach the vestiges

of the ossified areas (cf. Figs. 1B and 2F). We do not have an explanation for this phenotype, which is, however, more consistent with abnormal skeletogenesis than with the global patterning defects expected for *Hox* group *10* genes (Wellik and Capecchi 2003). Nevertheless, rib formation was not suppressed in *sm-Hoxa10* transgenic animals. Direct comparison of somitic *Hoxa10* expression levels by real-time RT–PCR indicates that higher *Hoxa10* somitic expression in *Dll1–Hoxa10* embryos cannot account for the qualitative differences in the phenotypes observed in the *Dll1–Hoxa10* and *sm-Hoxa10* transgenics (Supplemental Material). This is also supported by the clear phenotypic differences between the most affected *sm-Hoxa10* and the less affected *Dll1–Hoxa10* transgenics (Fig. 2; Supplemental Material). Altogether, these results indicate that the strong patterning effects observed in *Dll1–Hoxa10* transgenics did not result from *Hoxa10* expression in the somites but from its expression in the unsegmented paraxial mesoderm. Thus, *Hoxa10* confers specific patterning instructions to the somites in the presomitic mesoderm.

Hoxa11 *activity in the presomitic versus somitic mesoderm*

To investigate if this effect can be extended to other *Hox* genes, we performed a similar experiment with *Hoxa11*. We selected a gene of the *Hox11* group because, as for the *Hox10* group, the effects of its overexpression can be predicted; the *Hox11* group is essential for the genesis of sacral and caudal vertebrae (Wellik and Capecchi 2003), and thus, their overexpression is expected to produce signs of sacralization or caudalization at other levels of the axial skeleton. Ectopic expression of *Hoxa11* in the presomitic mesoderm (*Dll1–Hoxa11* transgenics) produced two main phenotypes in affected transgenics (five out of nine). First, at the thoracic level they all had multiple fusions between adjacent ribs (Fig. 3D,E). Since fusion of their lateral processes is a characteristic of sacral vertebrae, the alterations observed in the thoracic region of *Dll1–Hoxa11* transgenics can, indeed, be scored as partial sacralization. This is reminiscent of the sacral region of *Hox10*-null mutants, which show small ribs fused at their lateral margins, which has been interpreted as *Hox11* gene activity in the absence of *Hox10* genes (Wellik and Capecchi 2003). Thus, thoracic rib fusion is apparently a sign of *Hox11* activity in a *Hox10* negative area. The second general characteristic was an anteriorized position of the sacrum. In wild-type animals, the first sacral vertebra (S1) is vertebra number 27. In *Dll1–Hoxa11* transgenics, S1 was vertebra number 26 (one case), 25 (three cases), or 24 (one case) (Fig. 3B,C). In addition, cartilaginous lateral fusions characteristic of sacral vertebrae were observed between adjacent lumbar vertebrae (Fig. 3C) ($n = 2$), between adjacent caudal vertebrae (observed in the most affected transgenic) (Fig. 3F), and between adjacent vertebrae in the cervical area (two transgenics) (Fig. 3H). Finally, in two of the affected transgenics, anteriorly projecting protuberances resembling those observed in posterior sacral and caudal vertebrae emerged from the anterior lumbar vertebrae and replaced the rib in T13 (Fig. 3B).

To elucidate the contribution of the *Hoxa11* somitic activity to the *Dll1–Hoxa11* phenotype, we generated *sm-Hoxa11* transgenics, which express *Hoxa11* in the somites but not in presomitic mesoderm. None of the affected transgenic animals (four out of nine) showed the fusion between adjacent ribs that was observed in *Dll1–Hoxa11* transgenics, and their thoracic region conserved the general rib pattern. Instead, a clear and reproducible phenotype in the axial skeleton was observed in affected transgenic animals. In the lumbar area, all vertebrae contained anteriorly projecting lateral protuberances (Fig. 3J,K). As these protuberances were not fused, they can be considered a caudal rather than a sacral characteristic (Fig. 3I). Interestingly, the ribs also contained anteriorly projecting cartilaginous nodules close to their dorsal extremities (Fig. 3K), resembling the ectopic structures observed in the lumbar area, but inserted on the ribs. In addition, there was a clear tendency toward a shortening of the ossified area at the expense of the cartilaginous area, similar to that described for the *sm-Hoxa10* transgenics (Fig. 3K; data not shown). S1 was located at the appropriate axial level (vertebra 27) in three of the four affected transgenics. In the fourth, the sacrum was anteriorized unilaterally by one segment (Fig. 3L). Finally, no

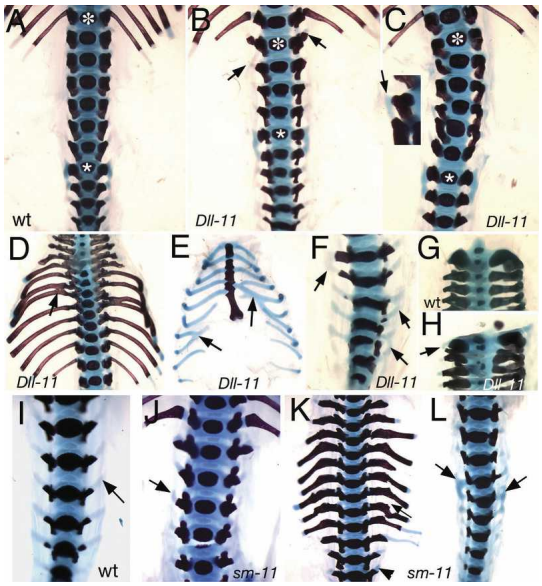

Figure 3. Patterning activity of *Hoxa11* expressed in the presomitic mesoderm and in the somites. (*A*) Ventral view of the lumbar and sacral area of a wild-type newborn. The ✻ indicates the position of vertebra 20, which is T13 in wild-type embryos; the ★ indicates S1. (*B*,*C*) Ventral views of two *Dll1–Hoxa11* transgenic embryos. In the embryo in *B*, S1 (★) is located in vertebra 24, and contains lateral protrusions in lumbar vertebrae (arrow). In the embryo in *C*, S1 is vertebra 26 and contains a lateral fusion between adjacent lumbar vertebrae (arrow in the blown-up region). (*D*) Thoracic area of a *Dll1–Hoxa11* transgenic newborn showing fusions between the ossified area of adjacent ribs (arrows). (*E*) Sternum and the cartilaginous area of the ribcage of a *Dll1–Hoxa11* transgenic newborn showing fusions between adjacent ribs (arrows). (*F*) Caudal region of a *Dll1–Hoxa11* transgenic newborn showing fusions between adjacent vertebrae (arrows). (*G*,*H*) Cervical area of a wild-type (*G*) and a *Dll1–Hoxa11* transgenic (*H*) newborn showing a lateral cartilaginous fusion between adjacent vertebrae (arrow in *H*). (*I*) Caudal area of a wild-type embryo. The arrow indicates the lateral process in a caudal vertebra. (*J–L*) The upper lumbar (*J*), thoracic (*K*), and sacral (*L*) areas of specific *sm-Hoxa11* transgenic newborns. The arrow in *J* indicates an anteriorly projecting protuberance in a lumbar vertebra. The arrow in *K* indicates an anteriorly projecting protuberance at the base of a rib, and the arrowhead indicates an anteriorly projecting protuberance in a lumbar vertebra. The arrows in *L* show the unilateral anteriorization of the sacrum.

caudal vertebra acquired sacral characteristics in *sm-Hoxa11* transgenics. Taken together, the above results indicate that the sacralization observed in *Dll1–Hoxa11* transgenics derived from *Hoxa11* expression in the presomitic mesoderm, and that the anteriorly projecting protuberance seen in T13 and the anterior lumbar area in two of these transgenics was probably due to residual *Hoxa11* expression in the somites. We conclude that, similar to what we found for the *Hox* group 10 genes, somites can acquire a *Hox* group 11 program when a gene of this group is expressed while somites are being formed.

Homeotic transformation in Gbx2 *mutant embryos*

In an independent study aimed at the identification of downstream targets of *Hoxa2* in the second branchial arch (Bobola et al. 2003; Kutejova et al. 2005), we identified *Gbx2* as a gene that is repressed by *Hoxa2* activity (Supplemental Material). When we analyzed the skeletal phenotype of *Gbx2* mutant embryos (Wassarman et al. 1997), we found that these embryos presented Hox-like homeotic transformation in the axial skeleton. In particular, they had 14 rib pairs (12 of 18 embryos) (Fig. 4C,D), indicating that L1 was transformed into a T14; eight ribs, instead of seven, were attached to the sternum (seven of 18 embryos), which indicates that T8 acquired T7 identity (Fig. 4E,F); and the transition vertebra was T11 instead of T10 (10 of 18 embryos) (Supplemental Material), another anterior transformation in the thoracic region. This result was completely unexpected because *Gbx2* is not expressed in the somites at any developmental stage (Fig. 4A,B; Bouillet et al. 1995; Wassarman et al. 1997; Supplemental Material). Interestingly, it is expressed in the presomitic mesoderm (Fig. 4A,B; Supplemental Material). *Gbx2* expression is broad in the posterior presomitic mesoderm with a diffuse anterior limit caudal to somitomere S0. The anterior expression border seems to be slightly more caudal at later developmental times when compared with younger embryos (Fig. 4B; Supplemental Material). Analysis of many embryos did not show evidence of a cycling behavior in the *Gbx2* expression in the presomitic mesoderm.

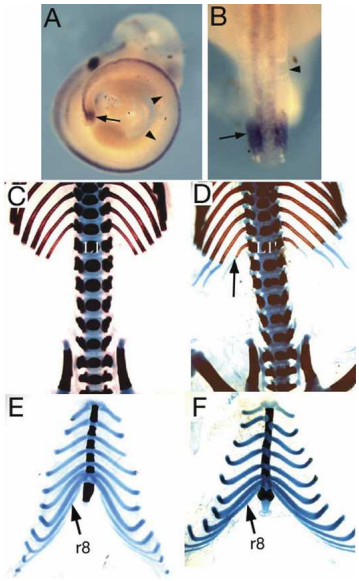

Figure 4. Axial skeletal phenotype of *Gbx2* mutant embryos. (*A*,*B*) Expression of *Gbx2* in E10.5 embryos by in situ hybridization. (*B*) A close-up look at the tail tip. It is expressed in the presomitic mesoderm (arrow) but not in the somites (arrowheads point to some of them). (*C*,*D*) Ventral view of the lower thoracic, lumbar, and sacral areas of a wild-type (*C*) and a *Gbx2* mutant (*D*) newborn. The sternum and the cartilaginous area of the ribcage were removed for clarity. The 21st vertebra, normally the first lumbar (L1), has an extra rib (arrow) in the *Gbx2* mutants. (*E*,*F*) Cartilaginous area of the ribcage of a wild-type (*E*) and a *Gbx2* mutant (*F*) newborn to show that the eighth rib (r8) is attached to the sternum in the *Gbx2* mutants but not in the wild-type embryos.

One possibility for the axial phenotype of *Gbx2*$^{-/-}$ embryos is that the absence of *Gbx2* resulted in the alteration of the anterior borders of *Hox* gene expression, similar to what has been previously described for mutants in the *Cdx* genes (Subramanian et al. 1995; Chawengsaksophak et al. 1997; van den Akker et al. 2002). Considering the strong similarity between the *Gbx2* and *Hoxc8* mutant phenotypes (Le Mouellic et al. 1992; van den Akker et al. 2001), we first examined *Hoxc8* expression to find no obvious alteration in its spatial–temporal expression in *Gbx2* mutant embryos (Supplemental Material). Phenotypes of mice containing specific mutations in the *Hox9* and *10* groups also shared some characteristics with the *Gbx2* mutant phenotype, particularly extra ribs in the lumbar area (Fromental-Ramain et al. 1996; Chen and Capecchi 1997; Wellik and Capecchi 2003). As for *Hoxc8*, we found no alterations in their anterior borders of somitic expression in *Gbx2* mutant embryos (Supplemental Material). These results indicate that other homeobox-containing genes (*Gbx2* also contains a homeobox) not belonging to the *Hox* clusters can provide segmental identity to the axial skeleton without affecting the anterior borders of somitic expression of genes within the *Hox* clusters. In addition, and most relevantly to the present study, the results support the finding that under physiological conditions, the activity of a homeobox-containing gene in the presomitic mesoderm is sufficient to provide patterning instructions to the resulting somites.

From our data it is not clear how *Gbx2* patterns the axial skeleton. As discussed above, it is highly unlikely that it does it by modulating Hox gene expression. Considering that *Gbx2* is a target of a *Hox* gene in the branchial area (Supplemental Material), one possibility is that it acts in the presomitic mesoderm downstream of another *Hox* gene. A good candidate is *Hoxc8*, as their mutant phenotypes in the axial skeleton are very similar. We are currently analyzing this possibility. An alternative hypothesis is that *Gbx2* does not function upstream or downstream of the *Hox* genes, but just modulates similar cellular/molecular processes as these genes. In fact, it also contains a homeobox.

Where do Hox *genes pattern the somites?*

The above transgenic experiments show that the patterning programs provided by *Hox* genes to the paraxial mesoderm may differ when they act during somite formation or in the already formed somites. For the *Hox10* group, the most relevant contribution to the morphogenesis of the axial skeleton seems to be already provided at the presomitic stage and not in the differentiating somites. The physiological role of expression of these

genes at later stages of somitic development has not been elucidated by our experiments. Our data do suggest that patterning by the *Hox11* group requires a combination of instructions given in the segmental plate and later in the somites. While formation of sacral structures is apparently instructed by the expression of *Hox* group *11* genes in the presomitic mesoderm, caudal vertebrae seem to require the activity of these genes in the somites. Interestingly, both areas are affected when all six *Hox* group *11* alleles are inactivated (Wellik and Capecchi 2003).

It is very likely that the prominent role of *Hox* gene expression in the presomitic mesoderm that we have observed in our transgenic animals is also relevant for the physiological *Hox* gene activity during axial patterning. This is supported by the finding that the expression of the *Hox* group *10* genes closely matches their functional domains at the stage when the relevant somites are being formed rather than at later stages, when somites start their differentiation programs. Also, at late developmental stages *Hox* group *11* genes seem to be active in somites fated to form caudal rather than sacral vertebrae (Burke et al. 1995). Our finding that inactivation of the *Gbx2* gene produced a typical *Hox* mutant phenotype in the axial skeleton without any apparent effect on *Hox* gene expression gives further support to the view that homeotic genes can provide patterning instructions in the presomitic mesoderm, as expression of this gene within the paraxial mesoderm is restricted to the segmental plate without any somitic contribution.

An important consequence from our findings is that understanding the patterning of the axial skeleton by *Hox* genes will require focusing the search for their target genes and respective mechanisms of activity to specific areas of the paraxial mesoderm. Interestingly, it has been shown that anomalous activity of the Notch, Wnt, and Fgf signaling pathways in the presomitic mesoderm also produces *Hox*-like transformations in the axial skeleton (Partanen et al. 1998; Ikeya and Takada 2001; Cordes et al. 2004). It has been suggested that the segmentation clock operating in the presomitic mesoderm could be linked to specific temporal activation of *Hox* genes, thus determining its appropriate anterior expression limit (Zakany et al. 2001). Our finding that *Hox* gene expression seems to commit the presomitic mesoderm opens an interesting, yet not exclusive, alternative. Thus, it could be hypothesized that *Hox* genes operate by modulating the response of the presomitic mesoderm to specific signaling inputs. In each segmentation cycle (Dale and Pourquie 2000), the segmentation signals operate on presomitic mesoderm expressing a particular combination of *Hox* genes, which would define specific patterns of response to the same signals, resulting in the formation of somites already containing specific patterning programs. In support of this, grafting experiments in chicken embryos have shown that presomitic mesoderm corresponding to a specific axial level transplanted to replace the presomitic mesoderm of a different level produces structures consistent with the position of the donor tissue (Kieny et al. 1972). Moreover, the ability of *Hox* genes to modulate the response of mesenchymal cells to Fgf signals has already been described in the craniofacial area (Bobola et al. 2003), and it could thus also be operative in the paraxial mesoderm. In light of this, it will be interesting to observe if stage-specific variations in the molecular cascades trigered by Notch, Fgf, and Wnt signals exist in the presomitic mesoderm and if they are affected by mutations in specific *Hox* genes.

Materials and methods

Transgenic and mutant animals

Transgenic constructs were generated using standard molecular biological techniques (Sambrook et al. 1989). The *Dll1* constructs contained the *Dll1* msd promoter (Beckers et al. 2000), the corresponding cDNAs, and the polyadenylation signal from SV40. The *sm* constructs contained a 2.5-kb BamHI fragment of the *Hoxa2* gene that includes the enhancer for rhombomere 2 (Frasch et al. 1995), the corresponding cDNAs, and the polyadenylation signal of SV40. The activity of the *sm* promoter was evaluated using the bacterial *tetR* gene as a reporter, whose expression was detected by whole-mount in situ hybridization using a *tetR*-specific probe (Mallo et al. 2003). The *Hoxa10* cDNA (mouse) was obtained from IMAGE clone 6511608. The *Hoxa11* cDNA (human) was obtained from IMAGE clone 5587615. Constructs were liberated from bacterial vector sequences, gel-purified, and used to generate transgenic embryos and animals by pronuclear injection according to standard protocols (Hogan et al. 1994).

The *Gbx2* mutant mice have been described before (Wassarman et al. 1997).

Molecular and phenotypic analyses

Whole-mount in situ hybridization was performed as described in Kanzler et al. (1998). The *Hoxa10* and *Hoxd10* probes were obtained from IMAGE clones 6511608 and 6516538, respectively. The *Hoxc10* probe was a 1091-bp fragment extending from position 29 to position 1119 of the mouse mRNA cloned by RT–PCR. The *Gbx2* probe was a 1.5-kb SmaI/XbaI fragment of the *Gbx2* cDNA.

Skeletal analyses were performed using the alcian blue/alizarin red staining method as described in Mallo and Brändlin (1997).

For transcript quantification, total RNA was isolated from dissected somites using TRI-Reagent (Roche), and first-strand cDNA was synthesized using random hexamer-primed reverse transcription. *Hoxa10* transcripts were then quantitated with the LightCycler (Roche) using the SYBR green PCR kit (QIAGEN) and primers 5′-AGCGAGTCCTAGA CTCCACGC-3′ and 5′-GTCCGTGAGGTGGACGCTACG-3′. *Gapdh* transcripts, amplified with primers 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, were used for normalization.

Acknowledgments

We thank B. Herrmann and A. Gossler for the *Dll1* promoter; G. Martin and A. Simeone for the *Gbx2* mice; J. Rodríguez-León and members of the Mallo lab for constant discussion; and R. Cassada, A. Coutinho, A. Jacinto, and J. Rodríguez-León for critically reading the manuscript. This work was supported by the Centro de Biologia do Desenvolvimento from FCT and by FCT/FEDER grant POCTI/MGI/43466/2001 to M.M.

References

Beckers, J., Caron, A., Hrabe de Angelis, M., Hans, S., Campos-Ortega, J.A., and Gossler, A. 2000. Distinct regulatory elements direct δ1 expression in the nervous system and paraxial mesoderm of transgenic mice. *Mech. Dev.* **95:** 23–34.

Bobola, N., Carapuco, M., Ohnemus, S., Kanzler, B., Leibbrandt, A., Neubuser, A., Drouin, J., and Mallo, M. 2003. Mesenchymal patterning by *Hoxa2* requires blocking Fgf-dependent activation of *Ptx1*. *Development* **130:** 3403–3414.

Bouillet, P., Chazaud, C., Oulad-Abdelghani, M., Dolle, P., and Chambon, P. 1995. Sequence and expression pattern of the Stra7 (Gbx-2) homeobox-containing gene induced by retinoic acid in P19 embryonal carcinoma cells. *Dev. Dyn.* **204:** 372–382.

Burke, A.C., Nelson, C.E., Morgan, B.A., and Tabin, C. 1995. *Hox* genes and the evolution of vertebrate axial morphology. *Development* **121:** 333–346.

Chawengsaksophak, K., James, R., Hammond, V.E., Kontgen, F., and Beck, F. 1997. Homeosis and intestinal tumours in Cdx2 mutant

mice. *Nature* **386:** 84–87.
Chen, F. and Capecchi, M.R. 1997. Targeted mutations in hoxa-9 and hoxb-9 reveal synergistic interactions. *Dev. Biol.* **181:** 186–196.
Cordes, R., Schuster-Gossler, K., Serth, K., and Gossler, A. 2004. Specification of vertebral identity is coupled to Notch signalling and the segmentation clock. *Development* **131:** 1221–1233.
Dale, K.J. and Pourquie, O. 2000. A clock-work somite. *Bioessays* **22:** 72–83.
Favier, B., Rijli, F.M., Fromental-Ramain, C., Fraulob, V., Chambon, P., and Dolle, P. 1996. Functional cooperation between the non-paralogous genes *Hoxa-10* and *Hoxd-11* in the developing forelimb and axial skeleton. *Development* **122:** 449–460.
Frasch, M., Chen, X., and Lufkin, T. 1995. Evolutionary-conserved enhancers direct region-specific expression of the murine *Hoxa-1* and *Hoxa-2* loci in both mice and *Drosophila*. *Development* **121:** 957–974.
Fromental-Ramain, C., Warot, X., Lakkaraju, S., Favier, B., Haack, H., Birling, C., Dierich, A., Dolle, P., and Chambon, P. 1996. Specific and redundant functions of the paralogous Hoxa-9 and Hoxd-9 genes in forelimb and axial skeleton patterning. *Development* **122:** 461–472.
Hirsinger, E., Jouve, C., Dubrulle, J., and Pourquie, O. 2000. Somite formation and patterning. *Int. Rev. Cytol.* **198:** 1–65.
Hogan, B., Beddington, R., Constantini, F., and Lacy, E. 1994. *Manipulating the mouse embryo: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Ikeya, M. and Takada, S. 2001. Wnt-3a is required for somite specification along the anteroposterior axis of the mouse embryo and for regulation of cdx-1 expression. *Mech. Dev.* **103:** 27–33.
Kanzler, B., Kuschert, S.J., Liu, Y.H., and Mallo, M. 1998. *Hoxa-2* restricts the chondrogenic domain and inhibits bone formation during development of the branchial area. *Development* **125:** 2587–2597.
Kessel, M. and Gruss, P. 1991. Homeotic transformations of murine vertebrae and concomitant alteration of *Hox* codes induced by retinoic acid. *Cell* **67:** 89–104.
Kieny, M., Mauger, A., and Sengel, P. 1972. Early regionalization of somitic mesoderm as studied by the development of axial skeleton of the chick embryo. *Dev. Biol.* **28:** 142–161.
Kondo, T. and Duboule, D. 1999. Breaking colinearity in the mouse HoxD complex. *Cell* **97:** 407–417.
Krumlauf, R. 1994. *Hox* genes in vertebrate development. *Cell* **78:** 191–201.
Kutejova, E., Engist, B., Mallo, M., Kanzler, B., and Bobola, N. 2005. Hoxa2 downregulates Six2 in the neural crest-derived mesenchyme. *Development* **132:** 469–478.
Le Mouellic, H., Lallemand, Y., and Brulet, P. 1992. Homeosis in the mouse induced by a null mutation in the Hox-3.1 gene. *Cell* **69:** 251–264.
Mallo, M. and Brändlin, I. 1997. Segmental identity can change independently in the hindbrain and rhombencephalic neural crest. *Dev. Dyn.* **210:** 146–156.
Mallo, M., Kanzler, B., and Ohnemus, S. 2003. Reversible gene inactivation in the mouse. *Genomics* **81:** 356–360.
Partanen, J., Schwartz, L., and Rossant, J. 1998. Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. *Genes & Dev.* **12:** 2332–2344.
Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Subramanian, V., Meyer, B.I., and Gruss, P. 1995. Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. *Cell* **83:** 641–653.
van den Akker, E., Fromental-Ramain, C., de Graaff, W., Le Mouellic, H., Brulet, P., Chambon, P., and Deschamps, J. 2001. Axial skeletal patterning in mice lacking all paralogous group 8 Hox genes. *Development* **128:** 1911–1921.
van den Akker, E., Forlani, S., Chawengsaksophak, K., de Graaff, W., Beck, F., Meyer, B.I., and Deschamps, J. 2002. Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* **129:** 2181–2193.
Wassarman, K.M., Lewandoski, M., Campbell, K., Joyner, A.L., Rubenstein, J.L., Martinez, S., and Martin, G.R. 1997. Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. *Development* **124:** 2923–2934.
Wellik, D.M. and Capecchi, M.R. 2003. *Hox10* and *Hox11* genes are required to globally pattern the mammalian skeleton. *Science* **301:** 363–367.
Zakany, J., Gerard, M., Favier, B., and Duboule, D. 1997. Deletion of a *HoxD* enhancer induces transcriptional heterochrony leading to transposition of the sacrum. *EMBO J.* **16:** 4393–4402.
Zakany, J., Kmita, M., Alarcon, P., de la Pompa, J.L., and Duboule, D. 2001. Localized and transient transcription of *Hox* genes suggests a link between patterning and the segmentation clock. *Cell* **106:** 207–217.